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Resensitization of the Desensitized Follicular Adenylyl Cyclase System to Luteinizing Hormone*

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ABSTRACT. LH-induced desensitization of the adenylyl cyclase system in a cell-free membrane preparation from preovulatory porcine follicles exhibits a critical dependence upon Mg and ATP (1). The membrane-rich preparation was found to contain endogenous cAMP-dependent and cAMP-independent protein kinases as well as phosphoprotein phosphatases. Endogenous phosphatase activity was enhanced by Mn^{2+} and dithiothreitol. The addition of either Mn^{2+} or dithiothreitol to the porcine follicular membrane preparation incubated under desensitizing conditions promoted a specific concentration-dependent reversal of the LH-induced desensitization of the adenylyl cyclase system. The addition of exogenous phosphoprotein phosphatase, partially purified from porcine follicular cytosol, also reversed LH-induced desensitization in a concentration-dependent manner. Boiling of the phosphatase preparation prevented reversal of desensitization. The addition of either exogenous beef heart cAMP-dependent protein kinase or heat-stable protein kinase inhibitor did not modify LH-induced desensitization of the follicular adenylyl cyclase system. These results provide indirect evidence that while LH-induced desensitization is not mediated by a cAMP-dependent protein kinase, reversal of desensitization can be promoted by activation of endogenous phosphatase and the addition of a homologous phosphatase preparation. (*Endocrinology* **104:** 1785, 1979)

WASHED membrane particles prepared from porcine ovarian follicles exceeding 6 mm in diameter have previously been shown to contain a highly LH-responsive adenylyl cyclase (AC) system which, upon incubation in the presence of LH, becomes unresponsive or desensitized to stimulation by LH (1). The desensitizing process required the presence of ATP and high Mg^{2+} (above 10 mM) or ATP, LH, and low Mg^{2+} (below 3 mM). Because of the critical Mg and ATP dependence of the desensitizing reaction, we felt that the desensitizing process may be mediated via a phosphorylation reaction. To this end, we investigated the possible role of a phosphorylation reaction in the AC-desensitizing process.

Materials and Methods

Materials

LH (lot NIH-LH-B9) was the generous gift of the NIH Hormone Distribution Officer (Bethesda, MD). AMP-P(NH)P was purchased from International Chemical and Nuclear Corp. (Irvine, CA). Creatine phosphate and creatine kinase were from Calbiochem (La Jolla, CA). Imidazole (Eastman) was purchased from Fisher Scientific Co. (Pittsburgh, PA), and aluminum oxide was obtained from Brinkmann Instruments (Des Plaines, IL). All other biochemical materials were purchased from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation mixture 3a-70B was purchased from Research Products International Corp. (Elk Grove Village, IL). [α-^{32}P]ATP, [γ-^{32}P]ATP, and [^{3}H]cAMP were purchased from New England Nuclear (Boston, MA).

Preparation of porcine follicular membrane particles and cytosol

Collection of ovaries, dissection of follicles exceeding 6 mm in diameter, and preparation of follicular membrane particles containing LH-stimulated AC activity were as described by Bockaert *et al.* (1). When a cytosol preparation was desired, the supernatant fraction derived from the first 12,000 × *g* centrifugation of the follicular homogenate was centrifuged at 105,000 × *g* for 60 min. The resulting supernatant fraction was designated cytosol.

DEAE-cellulose chromatography

For isolation of protein kinases, cytosol or the 12,000 × *g* washed membrane preparation (used for AC assays) containing approximately 10 mg protein was added to a column (1 × 3 cm) containing DEAE-cellulose (Whatmann, Inc., Clifton, NJ) initially equilibrated in 2 M NaCl at 4 C and reequilibrated just before use with 10 mM potassium phosphate, pH 7.0. Protein

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kinases were eluted from the column with a linear gradient of potassium phosphate buffer, pH 7.0, extending between 10–500 mM. Fractions were collected with a Gilson Micro fractionator (model FC 080 M, Gilson Medical Electronics, Inc., Middleton, WI) and 25-μl aliquots were assayed for protein kinase activity in the absence and presence of 4.5×10^{-7} M cAMP. Pretreatment of the porcine follicular membrane preparation with 0.1% Triton X-100 at (4 C for 1 h) did not alter the elution profile or recovery of protein kinases. Therefore, results shown are for the salt-extracted membrane protein kinases.

For the isolation of phosphatase from porcine follicular cytosol, cytosol containing approximately 60 mg protein was applied to a column (2 × 5 cm) of DEAE-cellulose. Phosphatase activity was eluted with a linear gradient of 10 mM tris-HCl and NaCl buffer extending from 0–500 mM NaCl, pH 7.0. Fractions were collected, as previously described, and were assayed for protein kinase and phosphatase activity. Phosphatase-containing fractions were pooled and further purified, as described in the following section. For the isolation of phosphatase from calf thymus nuclei, nonhistone proteins obtained from lysed nuclei by ammonium sulfate precipitation were subjected to DEAE-cellulose chromatography according to the technique described by Kranias *et al.* (2). Fractions containing phosphatase activity were pooled and purified further as described in the following section.

Partial purification of phosphatase-containing preparations from DEAE-cellulose chromatography

Phosphatase-containing preparations were further purified by a procedure adapted from that of Nakai and Thomas (3). After ammonium sulfate precipitation, the pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 50 mM β-mercaptoethanol. Removal of contaminating proteins was accomplished by successive freezing and thawing. β-Mercaptoethanol was then removed, and the preparation was concentrated by dialysis against 50 mM Tris-HCl, pH 7.4, and 25% glycerol, aliquoted, and stored at −70 C.

Preparation of heat-stable protein kinase inhibitor

A heat-stable inhibitor was prepared from rabbit skeletal muscle according to the method of Walsh *et al.* (4), as previously described (5).

AC assay

Membrane particles to be tested for their AC activity (15–50 μg membrane protein) were incubated in two- or three-stage reactions at 30 C in a final volume of 50 μl containing 1.75 or 2.0 mM [α-^{32}P]ATP (100–200 cpm/pmol); 6 mM $MgCl_2$; 1.0 mM EDTA; 1.0 mM EGTA; 1.0 mM [^{3}H]cAMP (approximately 8000 cpm); 25 mM 1,3-bis(Tris-[hydroxymethyl]methyl amine)-propane-HCl buffer, pH 7.0; and an ATP-regenerating system consisting of 0.2 mg/ml creatine kinase and 20 mM creatine phosphate. When LH-stimulated activity was determined, 0.625 μg LH in 1 mg/ml bovine serum albumin (BSA) was included in the first or last stage of the reaction mixture, as indicated below, to give a final concentration of 12.5 μg/ml LH. The two-stage reaction (method A), as described by Bockaert *et al.* (1), consisted first of a 20-min incubation in which membranes were incubated in a final volume of 40 μl with buffer, regenerating system, chelating agents, cAMP, $MgCl_2$, LH, and other additions (in concentrations 1.25 times final AC assay concentrations). The second stage of incubation (assay of AC activity) was started by the addition of 10 μl of a solution containing [α-^{32}P]ATP and other additions in the absence or presence of LH or other ingredients necessary for completion of assay conditions. The reaction was stopped after 5 min by the addition of 100 μl of a stopping solution containing 10 mM ATP, 10 mM cAMP, and 1% sodium dodecyl sulfate, followed immediately by boiling for 3 min.

The three-stage reaction (method B) consisted first of a 10-min incubation in which membranes were incubated in a final volume of 30 μl with the buffer, regenerating system, chelating agents, ATP, cAMP, and $MgCl_2$ in the absence or presence of LH (in concentrations 1.67 times final AC assay concentrations). The second stage of incubation was for 10 min and was started by the addition of 10 μl of a solution containing 1 mg/ml BSA or various additions. The third stage of incubation (AC assay) was started with the addition of 10 μl of a solution containing [α-^{32}P]ATP in the presence or absence of LH and was stopped after 5 or 6 min, as previously described for the two-stage reaction.

[^{32}P]cAMP formed by both incubation procedures was isolated using Dowex and alumina chromatography, as described by Bockaert *et al.* (1). Results are expressed in one of three ways: 1) as picomoles of cAMP formed during the 5- or 6-min AC assay per min/mg protein, 2) as the percent desensitization of the AC system when LH is present in all stages of the incubation reaction compared to when LH is present only in the last AC assay stage of the incubations, or 3) as the percent reversal of LH-induced desensitization of the LH-sensitive AC system, where 100% reversal is expressed by a fully LH-responsive AC system.

ATP levels were followed in some of the reactions by thin layer chromatography using polyethyleneimine-cellulose plates (Brinkmann Instruments, Inc., Wesbury, NY), as described by Birnbaumer *et al.* (6).

Protein kinase assay

Protein kinase activity was determined, as previously described (5), using protamine sulfate as substrate in the absence and presence of 4.5×10^{-7} M cAMP. ^{32}P-labeled product was isolated on Millipore filters (Millipore Corp., Bedford, MA) (5). Results are expressed as picomoles of ^{32}P-labeled product formed per min/mg protein.

Phosphatase assay

[^{32}P]Protamine chloride, substrate for the phosphatase assay, was prepared according to the technique described by Meisler and Langan (7), with minor modifications as follows. Namely, 10 mM cAMP, 2 mM theophylline, and 9.8 mM NaF were added to the reaction mixture to optimize the phosphorylation reaction, and a beef heart cAMP-dependent protein kinase (Sigma) was used as the enzyme source. [^{32}P]Protamine preparations were tested both for their alkali-labile and acid-labile phosphate content by the method of Meisler and Langan (7). The phos-

phate content was found to be 97–99% alkali labile and less than 1% acid labile.

Phosphatase activity was determined by measuring the release of [^{32}P]orthophosphate from [^{32}P]protamine chloride, according to the technique of Meisler and Langan (7). The enzyme concentrations were adjusted so that conversion rates were below 20% under these conditions with respect to label and time.

Protein was determined by the procedure of Lowry *et al.* (8), using crystalline BSA as standard.

Results

Preliminary findings

In preliminary experiments, we confirmed previous findings (1) which led to the suggestion that LH-induced desensitization of the LH-sensitive AC system may be mediated by a phosphorylation reaction. Specifically, desensitization (varying between 17–42% depending on individual membrane preparations) was obtained only when ATP (1–2 mM) and Mg (6 mM) were included in the desensitization stage of the incubation reaction; the omission of ATP from the desensitization stage and its inclusion only in the AC assay stage of the incubation reaction resulted in no desensitization of the AC system. Furthermore, when AMP-P(NH)P (1.8 mM), a nonphosphorylating analog of ATP which functions as a substrate for AC (9), was substituted for ATP in the desensitization stage of the incubation reaction, no desensitization of the LH-sensitive AC system was obtained.

Protein kinase and protein phosphatase activities in pig follicular membranes

Porcine follicular membranes which exhibit LH-responsive AC activity were found to contain both protein kinase and phosphoprotein phosphatase activities.

Of the protein kinase activity present in the membrane preparations, only 60% was cAMP-dependent, based upon the interaction of the heat-stable protein kinase inhibitor with the catalytic subunit of cAMP-dependent protein kinase (Table 1) (10). DEAE-cellulose chromatography of the membrane protein kinase activity revealed the presence of at least two different kinase enzymes (Fig. 1, *lower panel*). Analysis of the enzymes in the absence and presence of cAMP and before and after addition of the heat-stable protein kinase inhibitor showed peak I to be completely cAMP-independent and peak II, which eluted at the location of the type II variety (11–14), to be cAMP-dependent (Table 2). The elution profile of protein kinases obtained from pig follicular cytosol (Fig. 1, *upper panel*) was qualitatively similar to that of protein kinases from membranes; however, the low salt peak of protein kinase activity derived from the cytosol was not exclusively a cAMP-independent enzyme

TABLE 1. Protein kinase activity in porcine follicular membranes

Additions to protein kinase assay	Protein kinase activity (pmol/mg protein · min)	% Inhibition
−cAMP	171 ± 46	
+cAMP	389 ± 79	
+cAMP + inhibitor	147 ± 31	59 ± 3

Protein kinase activity was measured in the absence or presence of 4.5×10^{-7} M cAMP as well as in the presence of cAMP plus a saturating concentration of heat-stable protein kinase inhibitor (~6 μg protein) prepared from rabbit skeletal muscle. In separate experiments, this amount of inhibitor inhibited a commercially available (Sigma) beef heart protein kinase by 95%. Results are the mean ± SEM of 10 separate membrane preparations.

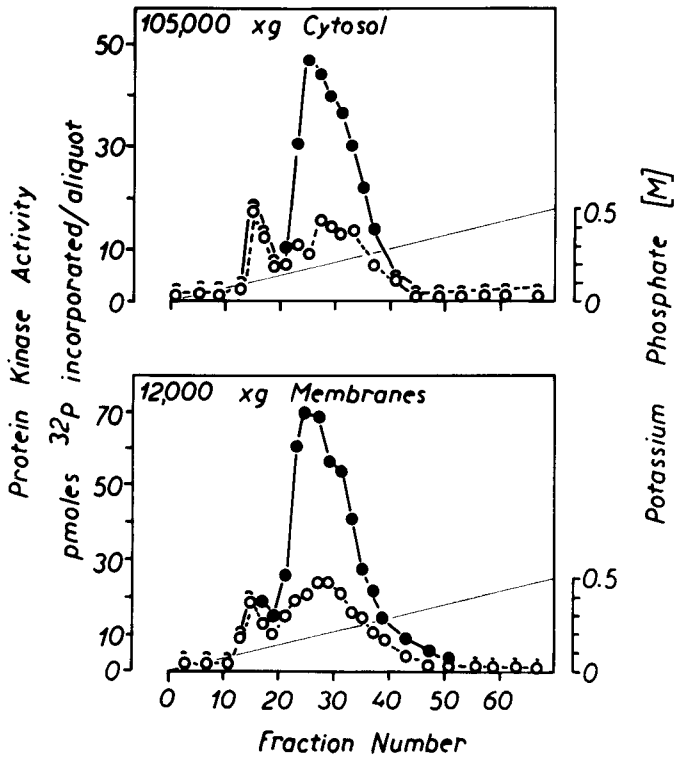

FIG. 1. DEAE-cellulose elution profiles of protein kinases derived from the 105,000 × *g* cytosol and a 12,000 × *g* washed membrane fraction of porcine follicles. The preparation and DEAE-cellulose chromatography of the cytosol and membrane fractions are described in *Materials and Methods*. Protein kinase activity was determined in the presence (●) and absence (○) of 4.5×10^{-7} M cAMP in 25-μl aliquots of 1-ml fractions eluted with a 10–500 mM potassium phosphate buffer. Representative elution profiles from a minimum of three experiments are shown.

as was that from the membranes (Table 2). Protamine, histone (lysine-rich fraction of type III-S), and casein [dephosphorylated by the technique of Ashby and Walsh (15)] were tested for their ability to serve as substrates for the membrane and cytosol protein kinases (not shown). Protamine was the preferred substrate of all kinases, while histone was a poor substrate for all kinases and was utilized 10% as efficiently as protamine. Casein

TABLE 2. Inhibition of porcine follicular membrane and cytosol protein kinases separated by DEAE-cellulose chromatography

Protein kinases	% Inhibition
105,000 × *g* Cytosol protein kinases	
Peak I	28 ± 7
Peak II	67 ± 8
12,000 × *g* Membrane protein kinases	
Peak I	0 ± 0
Peak II	71 ± 6

Peak I protein kinases eluted when the potassium phosphate concentration was ~0.1 M; peak II protein kinase eluted when the potassium phosphate concentration was ~0.2 M. Saturating concentrations of the heat-stable protein kinase inhibitor (6 μg protein) prepared from rabbit skeletal muscle were added to the protein kinase assay in the presence of 4.5×10^{-7} M cAMP. In each experiment, duplicate protein kinase determinations on the pooled fractions eluted from one DEAE-cellulose column were performed. Mean ± SD are shown where two or three such experiments were performed. For the rest of the conditions, see Table 1.

proved to be a better substrate only for the peak I protein kinases and was utilized by peak I enzymes 30% and 15%, respectively, for membrane *vs.* cytosol enzymes as efficiently as was protamine.

Porcine follicular membranes also contain endogenous phosphatase activity. Phosphatases associated with both membranes and cytosol exhibited pH optima of 6.8 (not shown). To determine the type of phosphatase present, we investigated the effects of various divalent metal cations on as well as potential substrates for the endogenous membrane- and cytosol-associated phosphatase activity, since acid, alkaline, and protein phosphatases each characteristically prefer different cations and substrates (16–19). Mn^{2+} strongly activated enzyme activities from both sources, being more effective on the cytosol preparation by a factor of 3, while Zn^{2+} markedly inhibited both (Table 3). The other divalent metal ions were without effect. Neither β-glycerol phosphate nor phosphoserine competed with protamine as a substrate for the phosphatases (Table 4). However, phosphatase activities from both preparations were severely inhibited by ATP and, to a lesser extent, by ADP. These characteristics along with the neutral pH preference suggest that the membrane and cytosol phosphatases are phosphoprotein as opposed to nonspecific acid or alkaline phosphatases.

Reversal of LH-induced desensitization of the LH-sensitive AC system

Effect of Mn^{2+} and dithiothreitol (DTT). Since Mn^{2+} (Table 2) (18) and DTT, routinely included in phosphatase assays, strongly activate endogenous follicular membrane phosphatases, we investigated the effect of these substances on the LH-sensitive AC system. Mn^{2+} promoted a concentration-dependent resensitization of the LH-sensitive AC system (Fig. 2). Neither basal (measured in the complete absence of LH) nor LH-stimulated AC activity (measured with LH present only during the 6-min AC assay) were significantly altered, but LH-stimulated desensitization (measured with LH present throughout the 20-min incubation and 6-min AC assay) was reversed. DTT, in concentrations less than 1 mM, also promoted a concentration-dependent reversal of desensitization, affecting only LH-stimulated desensitization and not basal or LH-stimulated AC activity (Fig. 3). Although the effects of Mn^{2+} and DTT that we observed on resensitization of the LH-sensitive AC system in porcine follicular membranes may well be due to activation of endogenous phosphatases, we cannot exclude the possibility that Mn^{2+} and DTT are directly modulating the AC system.

Effect of exogenously added phosphatase-containing preparations. In view of the indirect evidence for a role

TABLE 3. Effect of divalent metal ions on phosphatase activity in pig follicular cytosol and membrane preparations

Divalent metal ion	Relative activity (%)	
	Pig follicular cytosol	Pig follicular membranes
None	100	100
Mg^{2+}	110 ± 13	101 ± 2
Ca^{2+}	96 ± 5	106 ± 0
Mn^{2+}	929 ± 75	284 ± 14
Co^{2+}	82 ± 4	NT[a]
Zn^{2+}	7 ± 0	7 ± 1

Results are expressed as the mean ± SD of duplicate determinations of phosphatase activity determined on a washed 12,000 × *g* pellet obtained from pig follicles and on a phosphatase preparation partially purified from a 105,000 × *g* supernatant derived from pig follicles. The preparation of cytosol and membrane fractions, the procedure for partial purification of phosphatase from cytosol, and phosphatase assay are described in *Materials and Methods.* Final concentrations of the divalent metal ions were 2.5 mM.

[a] Not tested.

TABLE 4. Effect of various substrates on phosphatase activity in pig follicular cytosol and membrane preparations

Additions	Relative activity (%)	
	Pig follicular cytosol	Pig follicular membranes
None	100	100
β-Glycerol phosphate	91 ± 1	91 ± 5
Phosphoserine	117 ± 8	89 ± 2
ATP	42 ± 0	54 ± 1
ADP	56 ± 3	71 ± 7
AMP	126 ± 11	99 ± 11

Results are expressed as the mean ± SD of duplicate determinations Final concentrations of all additions are 2.5 mM. All procedures are as described in Table 3.

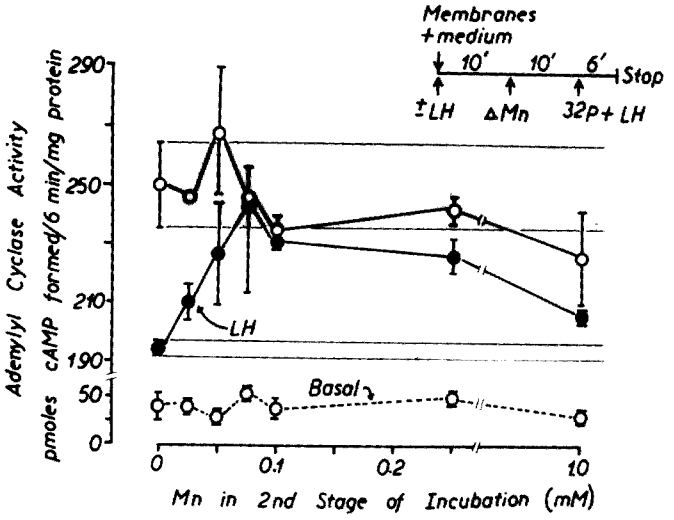

FIG. 2. Effect of Mn^{2+} on the AC activity of porcine follicular membranes. Graafian follicle membranes (18 μg protein) were incubated in three stages at 30 C, as diagramed on the figure (method B). In the first stage (10 min), 20 μl membrane were incubated in a volume of 30 μl in a medium consisting of buffer, $MgCl_2$, [^{3}H]cAMP, ATP, chelating agents, and the ATP-regenerating system in the presence of BSA or LH (all of which were at concentrations 1.67 times those desired in the third stage of the incubation). In the second stage of the incubation (10 min), Mn^{2+} (at concentrations indicated on the figure) was added to the first incubation mixture in a volume of 10 μl. In the third stage of incubation (AC assay), 10 μl of a medium consisting of [α-^{32}P] ATP and BSA or LH were added to the incubation mixture. Incubations were thus performed in the complete absence of LH (○- -○), with LH present in the 6-min AC assay only (○—○), or with LH present throughout the 26-min incubation time (●—●). The mean ± SD of duplicate determinations are shown.

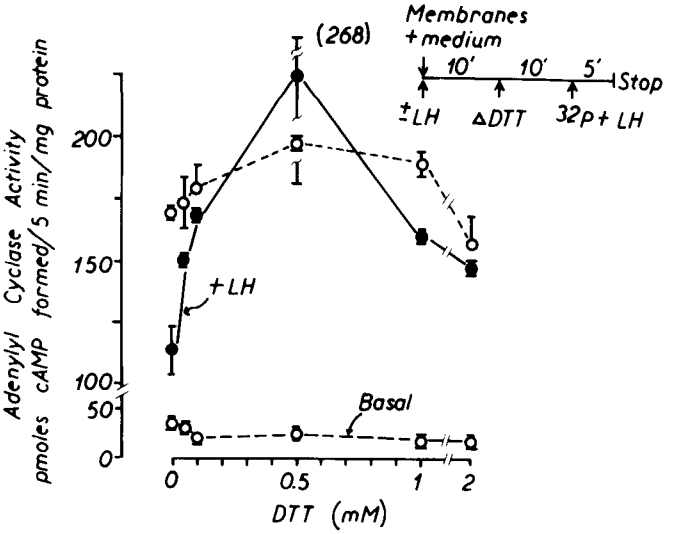

FIG. 3. Effect of DTT on AC activity of porcine follicular membranes (18 μg protein). Experimental protocol is as described in Fig. 2. DTT, in concentrations indicated on the figure, was added to the second stage of the incubation. The mean ± SD of duplicate determinations are shown.

of phosphatases in resensitization of the cyclase system provided by Mn^{2+}- and DTT-induced reversal of desensitization, we investigated the effects of various phosphatases on LH-induced desensitization in porcine follicular membranes. Incubation of the membranes with a phosphatase partially purified from pig follicle cytosol (Tables 3 and 4) reversed desensitization of the LH-sensitive AC system in a concentration-dependent manner (Fig. 4). Although LH-stimulated activity (measured with LH present only in the 5-min AC assay) was slightly increased, the major effect of the phosphatase-enriched preparation was on LH-stimulated desensitization (measured with LH present throughout the 25-min incubation). Boiling of the phosphatase-containing preparation at each concentration tested resulted in maximal desensitization (Fig. 5). In a separate experiment using a different batch of membranes and cytosol-derived phosphatase as well as slightly different incubation times, a phosphatase preparation (containing 35 μg protein and exhibiting a SA of 33 pmol/mg protein · min) reversed LH-stimulated desensitization by 57% (not shown). Complete reversal of LH-induced desensitization was obtained using a phosphatase-enriched preparation (containing 15 μg protein and exhibiting a SA of 110 pmol/mg protein · min) obtained from calf thymic nuclei (not shown). Experiments using various concentrations of commercially available alkaline phosphatases derived from human pla-

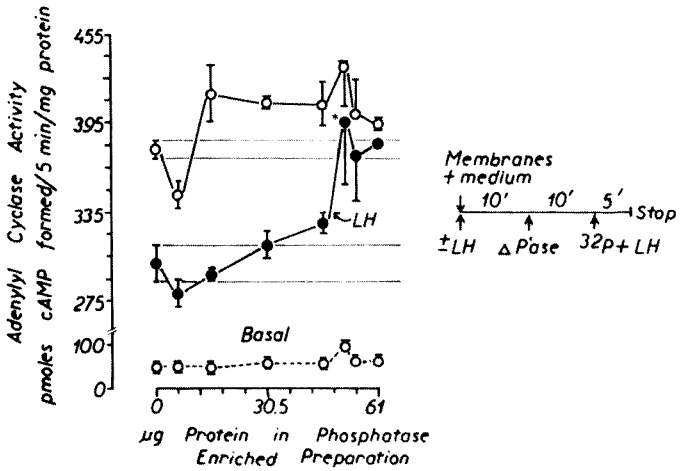

FIG. 4. Effect of phosphatase-enriched preparation on AC activity of porcine follicular membranes (28.5 μg protein). Experimental protocol is as described in Fig. 2. Protein phosphatase, partially purified from pig follicular cytosol (see Tables 3 and 4 for the effects of cations and potential substrates on the activity of this phosphatase) and free of protein kinase activity, was added to the second stage of the incubation in concentrations indicated on the figure. The mean ± SD of duplicate determinations are shown. *, $P < 0.05$ compared to the activity of the AC system determined in the presence of LH throughout the 25-min incubation and in the absence of exogenously added phosphatase.

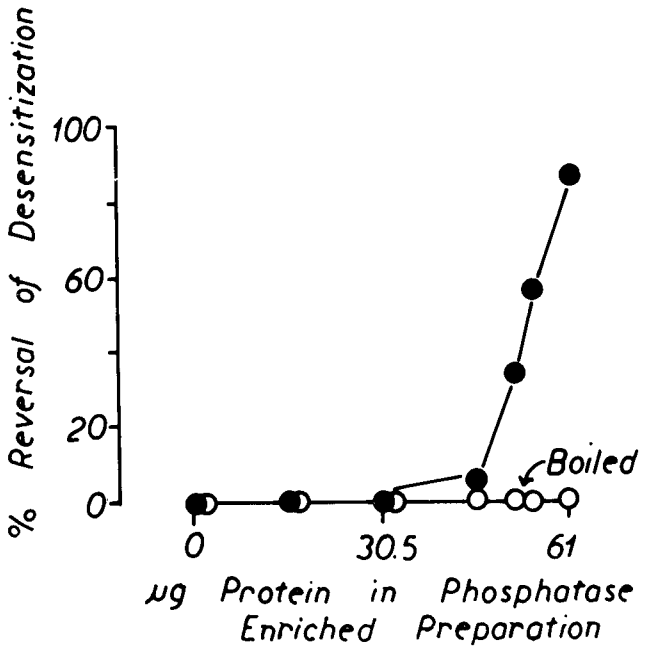

FIG. 5. Effect of intact and boiled phosphatase-enriched preparation on the reversal of desensitization. AC values using intact phosphatase are derived from the data in Fig. 4 (●). Equivalent concentrations of the phosphatase were boiled at 100 C for 20 min and then centrifuged at 10,000 × *g* for 20 min, and the supernatants were added to the second stage of the incubation, as described in Fig. 2. Percent desensitization is calculated as the decline in LH-stimulated AC activity measured with LH present in all three stages of the incubation *vs.* that with LH present only in the AC assay (stage 3). Zero percent reversal of desensitization corresponds to values obtained in the absence of added phosphatase (BSA control; 21% desensitization).

centa or calf intestine modified the AC system but did not reverse desensitization specifically by increasing the LH-stimulated desensitization (not shown). Addition of the phosphatase preparations to the last stage of AC incubation did not result in any detectable reversal of the desensitization phenomena (not shown).

Phosphatase-containing preparations were ineffective in hydrolyzing ATP to any of its metabolites either in incubations with [α-^{32}P]ATP alone or under AC assay conditions (Table 5). A decline in ATP levels during the AC incubation would promote an increase in the specific activity of ATP and an apparent decline in AC activity.

Effect of beef heart protein kinase and protein kinase inhibitor on LH-induced desensitization of the AC system. The ability of phosphoprotein phosphatase-rich preparations as well as Mn^{2+} and DTT (both of which can activate endogenous membrane protein phosphatases) to reverse LH-stimulated desensitization suggested that the resensitization of the cyclase system to LH stimulation may be mediated by a dephosphorylation reaction. It follows then that the reverse reaction, namely desensitization of the cyclase system, may be mediated by a phosphorylation reaction via protein kinase.

To this end, we investigated whether beef heart protein kinase, a type II cAMP-dependent protein kinase (13, 14), could enhance LH-induced desensitization. Concentrations of 0.5 and 1 μg (Fig. 6) and 0.05 μg (not shown) beef heart protein kinase did not ehance or reduce the extent of LH-induced desensitization. Higher concentra-

TABLE 5. Inability of phosphatase (P)-containing preparations to remove phosphate from ATP under various conditions

Condition	% of cpm as[a]			
	ATP	ADP	AMP	cAMP
[α-^{32}P]ATP	96.0	3.7	0.1	0.1
[α-^{32}P]ATP + P[b]	97.0	2.6	0.7	0.1
Control AC assay[c]	97.7	2.1	0.2	0.1
AC assay + P[d]	96.0	3.1	0.5	0.1
AC assay + boiled P[e]	98.0	1.3	0.1	0.2

[a] Percentages represent the percent of total added ^{32}P that chromatographed with an R_f equal to standard nucleotides on polyethyleneimine-cellulose thin layer sheets.

[b] [α-^{32}P]ATP was incubated for 30 min at 30 C in the presence of a phosphatase-containing preparation isolated from pig follicular cytosol. The phosphatase-containing preparation was present at a concentration 2.5 times greater than that in the AC assay.

[c] AC assay was performed using method A, as described in *Materials and Methods*, except that the assay samples were not boiled after the addition of the stopping solution. Between 1–2 μl incubation medium were applied to the polyethyleneimine-cellulose thin layer sheets. The percent distribution of nucleotides is the same regardless of whether basal or LH-stimulated AC activity is measured.

[d] Same AC assay as described in footnote *c* but with the addition of a phosphatase-containing preparation isolated from pig follicular cytosol which had been boiled for 20 min at 100 C.

[e] AC assay performed in the presence of a phosphatase-containing preparation isolated from pig follicular cytosol.

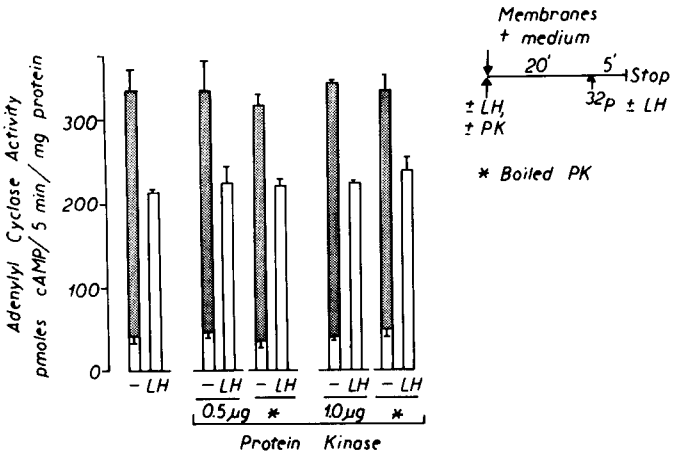

FIG. 6. Effect of beef heart protein kinase on AC activity of porcine follicular membranes (37 μg protein). Protocol is as described in Fig. 2. Protein kinase, in concentrations indicated on the figure, was added to the second stage of the incubation. Equivalent concentrations of protein kinase were boiled and treated as described in Fig. 5. — LH, BSA (—) or LH additions to first stage of incubation. ▨, LH added only to the last 5-min AC assay. The mean ± SD of duplicate determinations are shown.

tions of beef heart protein kinase (5 and 10 μg) interfered with the isolation of cAMP, as evidenced by a marked reduction in the recovery of [^{3}H]cAMP and presumably [^{32}P]cAMP from the Dowex and alumina columns.

We also tested the effect of the heat-stable protein kinase inhibitor on LH-induced desensitization of the AC system. If desensitization is mediated by a phosphorylation reaction catalyzed by a cAMP-dependent protein kinase, then the heat-stable protein kinase inhibitor should prevent LH-induced desensitization by virtue of the formation of the catalytically inactive complex between the heat-stable inhibitor and the catalytic subunit of cAMP-dependent protein kinase (10). Incubation of pig follicular membranes with varying concentrations of heat-stable protein kinase inhibitor failed to effect LH-induced desensitization of the LH-response AC system (Table 6). These latter two experiments suggest that desensitization is not mediated by a cAMP-dependent protein kinase, at least not by one of heterologous origin. This suggestion is further substantiated by the ability of LH to promote desensitization of the porcine follicular AC system in the absence of exogenous cAMP (1).

Discussion

After activation by tissue-specific hormones, the AC system often becomes unresponsive or desensitized to its own stimulant. The physiological effect of a desensitized AC system is a decline in cAMP levels and an inability of the tissue to respond to the hormonal stimulant. Desensitization of the AC system has been described in a great many other tissues as well as in the ovarian follicular system, as recently reviewed by Hunzicker-Dunn *et al.* (20).

However, in spite of the many descriptions of the AC-desensitizing process, the mechanism of hormone-induced desensitization is not yet known. There is convincing evidence in many systems to indicate that cyclase refractoriness is associated with a loss of hormone-specific membrane receptors (21–27). However, it is not clear from any of these studies if desensitization of the AC system is the cause or the result of receptor loss. Furthermore, utilizing porcine follicular membrane particles in which LH-induced desensitization occurs *in vitro* within 20 min, Bockaert *et al.* (1) found no evidence for a loss of receptors coincident with cyclase refractoriness.

In view of the absolute dependence upon Mg^{2+} and ATP of the desensitizing process in the porcine follicular cell-free membrane preparation, we investigated whether the activity of the AC system was modified by enzymes which mediate phosphorylation and dephosphorylation reactions. The following lines of indirect evidence indicate that the activity of the AC system may be partially regulated by the level of phosphorylation of one or more of the membrane-associated components of the AC system. 1) AC-rich membrane preparations contain both cAMP-dependent and cAMP-independent protein kinases as well as phosphoprotein phosphatases. The presence of both groups of enzymes are required if one is to suggest phosphorylation as a viable control mechanism for enzymatic regulation. 2) LH-induced desensitization exhibits an absolute dependence upon Mg^{2+} and ATP (1). 3) AMP-P(NH)P, a nonphosphorylating analog of ATP, cannot be substituted for ATP (1). 4) Both DTT and Mn^{2+}, shown capable of activating endogenous membrane protein phosphatases, specifically reverse LH-dependent desensitization in a concentration-dependent manner, rendering the AC system responsive to LH. 5) Protein phosphatase-enriched preparations also specifically reverse LH-dependent desensitization. 6) Nonspecific alkaline phosphatases do not specifically resensitize the desensitized AC system. This evidence indicates that resensitization is mediated by a dephosphorylation reaction. However, LH-induced desensitization does not appear to be mediated by a cAMP-dependent protein kinase. Neither beef heart protein kinase nor an inhibitor of protein kinase which complexes with endogenous catalytic subunits modified desensitization in any detectable manner. These conclusions on the lack of involvement of cAMP-dependent protein kinase in the desensitizing process are substantiated by results obtained by others using variants of the S 49 mouse lymphoma cell line which lack cAMP-dependent protein kinase (27). These investigators demonstrated equivalent isoproterenol-induced desensitization of the AC system in the cells which contained cAMP-dependent protein kinase and in the variants which lacked the protein kinase. Although these results demonstrate that desensitization of the AC system is not mediated by a cAMP-dependent protein ki-

TABLE 6. Lack of effect of heat-stable protein kinase inhibitor on desensitization of the AC

Inhibitor (μg protein)	% Desensitization
None	43.7 ± 3.5
0.6	41.1 ± 1.4
1.5	39.6 ± 2.5
3.0	38.5 ± 4.9
6.0	41.5 ± 2.9

Protein kinase inhibitor was added to the first stage of a two-stage incubation (method A) in which follicular membranes (48 μg protein) were incubated at 30 C for 20 min in a medium consisting of buffer, EDTA, EGTA, cAMP, ATP, and an ATP-regenerating system in the absence and presence of 12.5 μg/ml LH (at concentrations 1.25 times the final AC concentrations). The second stage of the incubation (AC assay) was started with the addition of 10 μl [α-^{32}P]ATP in the presence of BSA or 12.5 μg/ml LH and was terminated after 5 min. For the rest of the conditions, see *Materials and Methods*. Six micrograms of inhibitor protein isolated from rabbit skeletal muscle inhibited beef heart protein kinase by 96%. Results are expressed as the mean ± SD of triplicate determinations.

nase, we do not know if a cAMP-independent protein kinase can affect AC activity and are currently investigating this possibility.

The idea that the activity of the AC system is regulated by the level of membrane phosphorylation is not a new one. Naajar and Constantopolis (28–30) hypothesized that the AC system exists in an inactive phosphorylated form and in an active dephosphorylated form. They presented preliminary evidence that fluoride- and prostagladin E-stimulated activation of the AC system resulted in a 9–16% release of ^{32}P label, while the addition of protein kinase resulted in restoration of basal cyclase activity. Further evidence that the level of membrane phosphorylation may regulate AC activity is provided by the studies of Ho and Levey (31). These investigators found that glucagon stimulates the phosphorylation of specific membrane phospholipids which are reportedly associated with the coupling units between the receptor and catalytic subunits of the AC system.

Concrete proof that the activity of the AC system is regulated by a balance between protein kinase and phosphatase activities requires that phosphorylated forms of the AC system, or some component thereof, be found and that they be associated with the desensitized and activated states of the cyclase system.

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